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(54) Title: PROCESS FOR THE PREPARATION OF L-AMINO ACIDS BY USING CORYNEFORM BACTERIA

(57) Abstract: The invention relates to a process for the preparation of L-amino acids, wherein the following steps are implemented: a) fermentation of the coryneform bacteria producing the desired L-amino acid, in which at least the gene coding for 6-phosphofructokinase and/or the gene coding for 1-phosphofructokinase are/is attenuated, b) enrichment of the desired L-amino acid in the medium or in the cells of the bacteria, and c) isolation of the L-amino acid, and optionally bacteria are employed in which, in addition, further genes of the biosynthetic pathway of the desired L-amino acid are enhanced, or bacteria are employed in which the metabolic pathways that diminish the formation of the desired L-amino acid are at least partly switched off.

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# Process for the preparation of L-amino acids by using coryneform bacteria

## Field of the Invention

The invention provides a process for the preparation of L-amino acids, in particular L-lysine, by using coryneform bacteria in which the pfkA gene coding for 6-phosphofructokinase and/or the pfkB gene coding for 1-phosphofructokinase are/is attenuated.

## State of the Art

L-amino acids, in particular L-lysine, find application in human medicine and in the pharmaceutical industry, in the food industry and, quite especially, in animal nutrition.

It is known that amino acids are prepared by fermentation of strains of coryneform bacteria, in particular Corynebacterium glutamicum. On account of its great importance, work on improving the production process is constantly in progress. Improvements to the process may concern measures pertaining to fermentation technology, such as, for example, stirring and provision with oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during fermentation, or the reprocessing into product-form by, for example, ion-exchange chromatography, or the intrinsic output properties of the micro-organism itself.

With a view to improving the output properties of these micro-organisms, methods of mutagenesis, selection and mutant selection are adopted. In this way, strains are obtained that are resistant to antimetabolites such as, for example, the lysine analogue S-(2-aminoethyl)cysteine or that are auxotrophic in respect of metabolites of regulatory significance and that produce L-amino acids.

Methods pertaining to recombinant DNA technology have also been employed for a number of years for the improvement of strains of Corynebacterium glutamicum producing L-amino acid, by individual amino-acid-biosynthesis genes being amplified and by the effect on the production of L-amino acid being investigated.

Object of the Invention

The inventors have set themselves the task of making available new foundations for improved processes for the fermentative preparation of L-amino acids, in particular L-lysine, by using coryneform bacteria.

Summary of the Invention

When mention is made in the following of L-amino acids or amino acids, these expressions are intended to mean one or more amino acids, including their salts, selected from the group comprising L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-lysine is particularly preferred.

When mention is made in the following of L-lysine or lysine, these expressions are intended to mean not only the bases but also the salts such as, for example, lysine monohydrochloride or lysine sulfate.

The invention provides a process for the fermentative preparation of L-amino acids by using coryneform bacteria in which at least the nucleotide sequence coding for 6-phosphofructokinase and/or the nucleotide sequence coding for 1-phosphofructokinase are/is attenuated, in particular switched off or expressed at a low level.

This invention further provides a process for the fermentative preparation of L-amino acids in which the following steps are implemented:

a) fermentation of the coryneform bacteria producing the L-amino acid, in which at least the nucleotide sequence coding for 6-phosphofructokinase and/or the nucleotide sequence coding for 1-phosphofructokinase are/is attenuated, in particular switched off or expressed at a low level;

b) enrichment of the L-amino acids in the medium or in the cells of the bacteria; and

c) isolation of the desired L-amino acids, whereby constituents of the fermentation broth and/or of the biomass optionally remain in the end product in proportions or in their total quantities.

Detailed Description of the Invention

The strains that are employed preferably already produce L-amino acids, in particular L-lysine, before the attenuation of the pfkA gene coding for 6-phosphofructokinase and/or of the pfkB gene coding for 1-phosphofructokinase.

Preferred embodiments are to be found in the Claims.

The term 'attenuation' in this context describes the diminution or switching-off of the intracellular activity of one or more enzymes (proteins) in a micro-organism that are coded by the corresponding DNA, by use being made, for example, of a weak promoter or by use being made of a gene or allele that codes for a corresponding enzyme with a low activity or that inactivates the corresponding gene or enzyme (protein) and by these measures optionally being combined.

By virtue of the measures of attenuation, the activity or concentration of the corresponding protein is lowered in general to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the initial micro-organism.

The micro-organisms that are the subject-matter of the present invention are able to produce amino acids from glucose, saccharose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerin and ethanol. It may be a question of representatives of coryneform bacteria, in particular of the genus Corynebacterium. In the case of the genus Corynebacterium, in particular the species Corynebacterium glutamicum should be mentioned, which is known amongst experts for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum, are especially the known wild-type strains

Corynebacterium glutamicum ATCC13032

Corynebacterium acetoglutamicum ATCC15806

Corynebacterium acetoacidophilum ATCC13870

Corynebacterium melassecola ATCC17965

Corynebacterium thermoaminogenes FERM BP-1539

Brevibacterium flavum ATCC14067

Brevibacterium lactofermentum ATCC13869 and

Brevibacterium divaricatum ATCC14020

and mutants and strains prepared therefrom that produce L-amino acids,

such as, for example, the L-lysine-producing strains

Corynebacterium glutamicum FERM-P 1709

Brevibacterium flavum FERM-P 1708

Brevibacterium lactofermentum FERM-P 1712

Corynebacterium glutamicum FERM-P 6463

Corynebacterium glutamicum FERM-P 6464 and

Corynebacterium glutamicum DSM 5715.

It has been found that coryneform bacteria produce L-amino acids in improved manner after attenuation of the gene coding for 6-phosphofructokinase (EC: 2.7.1.11) and/or of the gene coding for 1-phosphofructokinase (EC 2.7.1.56).

The nucleotide sequence of the gene coding for 6-phosphofructokinase of Corynebacterium glutamicum can be gathered from patent application WO 01/00844 under Identification Code RXA00206 as SEQ ID No. 53.

The nucleotide sequence of the gene coding for 1-phosphofructokinase of Corynebacterium glutamicum can be gathered from patent application WO 01/00844 under Identification Code RXA01882 as SEQ ID No. 57.

The nucleotide sequences are also deposited in the gene bank under Accession Numbers AX064927 and AX064931, respectively.

The claimed nucleotide sequences of the genes coding for 1-phosphofructokinase and for 6-phosphofructokinase, represented in SEQ ID No. 3 and SEQ ID No. 1, respectively, are elongated in comparison with the sequences known from the state of the art by, in each instance, preferably up to 700 base-pairs in front of the start codon and behind the stop codon of the gene.

The elongations in comparison with the sequence known from the state of the art consist in SEQ ID No. 3 of the base-pairs 1 to 508 and 1684 to 2234, respectively.

In SEQ ID No. 1 the elongations in comparison with the sequence known from the state of the art consist of the base-pairs 1 to 531 and 1621 to 2160, respectively.

The amino-acid sequences of the associated gene products are represented in SEQ ID No. 4 and SEQ ID No. 2, respectively.

It has been found that processes for attenuation that are known as such can be employed particularly successfully with the aid of the elongated sequences that are made available in this way.

Such a process is the method of gene replacement. With this method, a mutation such as, for example, a deletion, an insertion or base-exchange in the gene of interest is produced in vitro. The allele that is produced is, in turn, cloned into a vector that is non-replicative in respect of C. glutamicum and said vector is subsequently converted by transformation or conjugation into the desired host of C. glutamicum. After homologous recombination by means of a first cross-over event bringing about integration and by means of a suitable second cross-over event in the target gene or in the target sequence bringing about an excision, the incorporation of the mutation or of the allele is obtained. This method was used in EP: 00110021.3, for example, in order to switch off the secG gene of C. glutamicum.

The elongation of the sequences that are employed is not restricted to 600 base-pairs in front of the start codon and behind the stop codon. It preferably lies within the range from 300 to 700 base-pairs, but it may also amount to

up to 800 base-pairs. The elongations may also contain different quantities of base-pairs.

The sequences, described in the stated passages, coding for 6-phosphofructokinase or 1-phosphofructokinase can be used in accordance with the invention. Moreover, use can be made of alleles of 6-phosphofructokinase or 1-phosphofructokinase that arise from the degenerate nature of the genetic code or as a result of functionally neutral sense mutations.

With a view to achieving an attenuation, either the expression of the gene coding for 6-phosphofructokinase and/or of the gene coding for 1-phosphofructokinase or the catalytic properties of the gene products can be lowered or switched off. Both measures are optionally combined.

The gene expression can be reduced by suitable culturing or by genetic modification (mutation) of the signal structures of gene expression. Signal structures of gene expression are, for example, repressor genes, activator genes, operators, promoters, attenuators, ribosome binding sites, the start codon and terminators. Data relating to this can be found by a person skilled in the art in, for example, patent application WO 96/15246, in Boyd and Murphy (Journal of Bacteriology 170: 5949 (1988)), in Voskuil and Chambliss (Nucleic Acids Research 26: 3548 (1998), in Jensen and Hammer (Biotechnology and Bioengineering 58: 191 (1998)), in Pátek et al. (Microbiology 142: 1297 (1996)) and in known textbooks on genetics and molecular biology such as, for example, the textbook by Knippers (*Molekulare Genetik*, 6th Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995) or in that by Winnacker (*Gene und Klone*, VCH Verlagsgesellschaft, Weinheim, Germany, 1990).

Mutations that lead to a change in or a lowering of the catalytic properties of enzyme proteins are known from the state of the art; by way of examples, mention may be made

of the papers by Qiu and Goodman (Journal of Biological Chemistry 272: 8611-8617 (1997)), Sugimoto et al. (Bioscience Biotechnology and Biochemistry 61: 1760-1762 (1997)) and Möckel (*Die Threonindehydratase aus Corynebacterium glutamicum: Aufhebung der allosterischen Regulation und Struktur des Enzyms*, Berichte des Forschungszentrums Jülichs, Jül-2906, ISSN09442952, Jülich, Germany, 1994). Synoptic accounts can be gathered from known textbooks on genetics and molecular biology such as, for example, that by Hagemann (*Allgemeine Genetik*, Gustav Fischer Verlag, Stuttgart, 1986).

Transitions, transversions, insertions and deletions enter into consideration by way of mutations. Depending on the effect of the amino-acid exchange on the enzyme activity, one speaks of missense mutations or nonsense mutations. Insertions or deletions of at least one base-pair in a gene lead to frame-shift mutations, as a consequence of which false amino acids are incorporated or the translation terminates prematurely. Deletions of several codons typically lead to a complete loss of enzyme activity. Instructions for the generation of mutations of such a type pertain to the state of the art and can be gathered from known textbooks on genetics and molecular biology such as, for example, the textbook by Knippers (*Molekulare Genetik*, 6$^{th}$ Edition, Georg Thieme Verlag, Stuttgart, Germany, 1995), that by Winnacker (*Gene und Klone*, VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann (*Allgemeine Genetik*, Gustav Fischer Verlag, Stuttgart, 1986).

Customary methods for mutating genes of C. glutamicum are the methods of gene disruption and of gene replacement described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

In the case of the method of gene disruption, a central part of the coding region of the gene of interest is cloned

into a plasmid vector that is able to replicate in a host (typically E. coli) but not in C. glutamicum. By way of vectors, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992)), pGEM-T (Promega corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994), Journal of Biological Chemistry 269:32678-84; US Patent 5,487,993), pCR®Blunt (Invitrogen, Groningen, Netherlands; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) or pEM1 (Schrumpf et al, 1991, Journal of Bacteriology 173:4510-4516), for example, enter into consideration. The plasmid vector, which contains the central part of the coding region of the gene, is subsequently converted by conjugation or transformation into the desired strain of C. glutamicum. The method of conjugation is described, for example, in Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a cross-over event, the coding region of the gene in question is interrupted by the vector sequence, and two incomplete alleles are obtained, from each of which the 3'-end or the 5'-end is missing. This method was used, for example, by Fitzpatrick et al. (Applied Microbiology and Biotechnology 42, 575-580 (1994)) for the purpose of switching off the recA gene of C. glutamicum.

In the case of the method of gene replacement, a mutation such as, for example, a deletion, an insertion or a base-exchange in the gene of interest is produced in vitro. The allele that is produced is, in turn, cloned into a vector that is non-replicative in respect of C. glutamicum and

said vector is subsequently converted by transformation or conjugation into the desired host of C. glutamicum. After homologous recombination by means of a first cross-over event bringing about integration and by means of a suitable second cross-over event in the target gene or in the target sequence bringing about an excision, the incorporation of the mutation or of the allele is obtained. This method was used, for example, by Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998)), in order to switch off the pyc gene of C. glutamicum by a deletion.

In this way a deletion, an insertion or a base-exchange can be incorporated into the gene coding for 6-phosphofructokinase and/or the gene coding for 1-phosphofructokinase.

In addition, for the production of L-amino acids it can be advantageous, in addition to the attenuation of the gene coding for 6-phosphofructokinase and/or of the gene coding for 1-phosphofructokinase, to enhance, in particular to overexpress, one or more enzymes of the respective biosynthetic pathway, of glycolysis, of anaplerotic reactions, of the citric-acid cycle, of the pentose-phosphate cycle, of the export of amino acid and optionally regulatory proteins.

The term 'enhancement' or in this context describes the increase in the intracellular activity of one or more enzymes or proteins in a micro-organism which are coded by the corresponding DNA by, for example, the copy-number of the gene or genes being increased, by use being made of a strong promoter or a gene that codes for a corresponding enzyme or protein with a high activity and by optionally combining these measures.

By virtue of the measures of enhancement, in particular overexpression, the activity or concentration of the corresponding protein is increased in general by at least

10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, maximally up to 1000% or 2000%, relative to that of the wild-type protein or of the activity or concentration of the protein in the initial micro-organism.

The use of endogenous genes is generally preferred. The term "endogenous genes" or "endogenous nucleotide sequences" is to be understood to mean the genes or nucleotide sequences, respectively, existing in the population of a species.

Thus, for the preparation of L-lysine, in addition to the attenuation of the gene coding for 6-phosphofructokinase and/or of the gene coding for 1-phosphofructokinase, one or more of the genes selected from the group comprising

- the gene lysC coding for a feedback-resistant aspartate kinase (Accession No. P26512, EP-B-0387527; EP-A-0699759; WO 00/63388),
- the gene dapA coding for dihydrodipicolinate synthase (EP-B 0 197 335),
- the gene gap coding for glyceraldehyde-3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- simultaneously the gene pyc coding for pyruvate carboxylase (DE-A-198 31 609),
- the gene mqo coding for malate:quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395 - 403 (1998)),
- the gene zwf coding for glucose-6-phosphate dehydrogenase (JP-A-09224661),
- simultaneously the gene lysE coding for the lysine-export protein (DE-A-195 48 222),

- the gene zwa1 coding for the zwa1 protein (DE: 19959328.0, DSM 13115),

- the gene tpi coding for triosephosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086), and

- the gene pgk coding for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086)

is/are enhanced, in particular overexpressed.

Moreover, for the production of amino acids, in particular L-lysine, it can be advantageous, in addition to the attenuation of the gene coding for 6-phosphofructokinase and/or of the gene coding for 1-phosphofructokinase, simultaneously to attenuate, in particular to reduce the expression of, one or more of the genes selected from the group comprising

- the gene pck coding for phosphoenolpyruvate carboxykinase (DE 199 50 409.1, DSM 13047),

- the gene pgi coding for glucose-6-phosphate isomerase (US 09/396,478, DSM 12969),

- the gene poxB coding for pyruvate oxidase (DE:1995 1975.7, DSM 13114),

- the gene fda coding for fructose bisphosphate aldolase (Mol. Microbiol. 3 (11), 1625-1637 (1989); gene bank Accession Number X17313) and

- the gene zwa2 coding for the zwa2 protein (DE: 19959327.2, DSM 13113).

Finally, for the production of amino acids it can be advantageous, in addition to the attenuation of the gene coding for 6-phosphofructokinase and/or of the gene coding

for 1-phosphofructokinase, to exclude undesirable side reactions (Nakayama: *Breeding of Amino Acid Producing Micro-organisms*, in: *Overproduction of Microbial Products*, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The micro-organisms that are produced in accordance with the invention are likewise a subject of the invention and can be cultivated continuously or discontinuously in the batch process (batch cultivation) or in the fed-batch or repeated-fed-batch process for the purpose of producing L-amino acids. A summary of known cultivation methods is described in the textbook by Chmiel (*Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik* (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (*Bioreaktoren und periphere Einrichtungen* (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used has to satisfy the demands of the respective strains in suitable manner. Descriptions of culture media of various micro-organisms are contained in the manual entitled *Manual of Methods for General Bacteriology* published by the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugar and carbohydrates such as, for example, glucose, saccharose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats such as, for example, soybean oil, sunflower oil, peanut oil and coconut oil, fatty acids such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols such as, for example, glycerin and ethanol, and organic acids such as, for example, acetic acid can be used by way of carbon source. These substances can be used individually or as a mixture.

Organic nitrogenous compounds such as peptones, yeast extract, meat extract, malt extract, maize steep liquor, soybean flour and urea, or inorganic compounds such as

ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate can be used by way of nitrogen source. The nitrogen sources can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium-containing salts can be used by way of phosphorus source. The culture medium must, moreover, contain salts of metals such as, for example, magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential growth substances such as amino acids and vitamins can be employed in addition to the aforementioned substances. Besides, suitable precursors can be added to the culture medium. The stated feed materials can be added to the culture in the form of a single charge or can be fed in during the cultivation in suitable manner.

With a view to controlling the pH of the culture, basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammoniacal liquor or acidic compounds such as phosphoric acid or sulfuric acid are employed in suitable manner. With a view to controlling the formation of foam, anti-foaming agents such as, for example, fatty-acid polyglycol esters can be employed. With a view to maintaining the stability of plasmids, suitable substances acting selectively, such as antibiotics for example, can be added to the medium. In order to maintain aerobic conditions, oxygen or oxygenous gas mixtures, such as air for example, are introduced into the culture. The temperature of the culture is normally around 20°C to 45°C and preferably 25°C to 40°C. The culture is continued for such time until a maximum of the desired product has formed. This objective is normally attained within a period from 10 hours to 160 hours.

Methods for the determination of L-amino acids are known from the state of the art. The analysis can be undertaken

as described in Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion-exchange chromatography with subsequent ninhydrin derivation, or it can be undertaken by reversed-phase HPLC, as described in Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The following micro-organism was deposited in the form of pure culture on 11 January 2002 in the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Micro-Organisms and Cell Cultures, DSMZ, Braunschweig, Germany):

- Escherichia coli DH5alphamcr/pXK99EmobpfkB (= DH5αmcr/pXK99EmobpfkB) as DSM 14741.

The present invention is elucidated in more detail in the following on the basis of exemplary embodiments.

Example 1

Preparation of a genomic cosmid gene bank from Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described in Tauch et al. (1995, Plasmid 33:168-179) and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, Code No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Code No. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, product description SuperCos1 Cosmid Vektor Kit, Code No. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, product description XbaI, Code No. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

Subsequently the cosmid DNA was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, Code No. 27-0868-04). The cosmid DNA that was treated in this way was mixed with the treated ATCC13032 DNA, and the charge was treated with T4-DNA-Ligase (Amersham Pharmacia, Freiburg, Germany, product description T4-DNA-Ligase, Code No. 27-0870-04). The ligation mixture was subsequently packaged in phages with the aid of the Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, product description Gigapack II XL Packing Extract, Code No. 200217).

With a view to infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575), the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. Infection and titration

of the cosmid bank were carried out as described in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), whereby the cells were plated onto LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. After incubation overnight at 37°C, recombinant single clones were selected.

Example 2

Ascertainment of the upstream and downstream elongations of the sequences of the genes pfkA and pfkB known from the state of the art

The cosmid DNA of a single colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Product No. 1758250). After gel-electrophoretic fractionation, isolation of the cosmid fragments was effected within the size-range from 1500 to 2000 bp with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1 obtained from Invitrogen (Groningen, Netherlands, product description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments into the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), whereby the DNA mixture was incubated overnight with T4-Ligase (Pharmacia Biotech, Freiburg, Germany). This ligation

mixture was subsequently electroporated into the E. coli strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) and plated onto LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l Zeocin.

The plasmid preparation of the recombinant clones was undertaken with a Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). Sequencing was effected in accordance with the dideoxy chain-termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) with modifications in accordance with Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). Use was made of the "RR dRhodamin Terminator Cycle Sequencing Kit" manufactured by PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany). Gel-electrophoretic fractionation and analysis of the sequencing reaction were undertaken in a "Rotiphorese NF Acrylamid/Bisacrylamid" gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with an "ABI Prism 377" sequencer manufactured by PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were subsequently processed by applying the Staden program package (1986, Nucleic Acids Research, 14:217-231) Version 97-0. The individual sequences of the pZero1 derivatives were assembled into a coherent contig. The computer-aided coding-region analysis was produced with the program XNIP (Staden, 1986, Nucleic Acids Research, 14:217-231).

The known nucleotide sequences of the genes pfkA and pfkB that are extended by the upstream and downstream elongations obtained in this way are represented in SEQ ID No. 1 and SEQ ID No. 3.

Example 3

Preparation of the expression vector pXK99EmobpfkB for IPTG-induced expression of the pfkB gene in C. glutamicum

3.1 Cloning of the pfkB gene

Chromosomal DNA is isolated from the strain ATCC 13032 in accordance with the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)). On the basis of the known sequence of the pfkB gene for C. glutamicum the following oligonucleotides for the polymerase chain reaction are selected (see SEQ ID No. 5 and SEQ ID No. 6):

pfkB for:
5`-CT TCT AGA-CCC GAC CAC AAC TTT CAG G -3`

pfkBint int:
5`- AG AAG CTT-GCC AGG TGT ATC CAA GCT CTC -3`

In this connection the primers are selected in such a way that the amplified fragment contains the incomplete gene, beginning with the native ribosome binding site without promoter region, as well as the anterior region of the pfkB gene. In addition, the primer pfkB for contains the sequence for the cleavage site of the restriction endonuclease XbaI, and the primer pfkB int contains the sequence for the cleavage site of the restriction endonuclease HindIII, which are marked by underlining in the nucleotide sequences represented above.

The primers that are represented are synthesised by MWG-Biotech AG (Ebersberg, Germany), and the PCR reaction is carried out in accordance with the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press) with Pwo polymerase produced by Roche Diagnostics GmbH (Mannheim, Germany). With the aid of the polymerase chain reaction the primers enable the amplification of a 594-bp DNA fragment that

bears the incomplete pfkB gene including the native ribosome binding site.

The 594-bp pfkB fragment is cleaved with the restriction endonucleases XbaI and HindIII and is subsequently isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

3.2 Construction of the expression vector pXK99Emob

The IPTG-inducible expression vector pXK99Emob is constructed in accordance with the state of the art. The vector is based on the Escherichia coli expression vector pTRC99A (Amann et al., Gene 69: 301-315 (1988)) and contains the trc promoter which is inducible by addition of the lactose derivative IPTG (isopropyl-β-D-thiogalactopyranoside), the termination regions T1 and T2, the replication origin ColE1 from E. Coli, the $lacI^q$ gene (repressor of the lac operon of E.coli), a multiple cloning site (mcs) (Norrander, J.M. et al. Gene 26, 101-106 (1983)), the kanamycin-resistance gene aph(3´)-IIa from E. coli (Beck et al. (1982), Gene 19: 327-336) and the RP4 mobilization site from the cloning vector pK18mobsacB (Schäfer et al., Gene 14: 69-73 (1994)).

It has been found that the vector pXK99Emob is quite especially suitable for regulating the expression of a gene, in particular for bringing about the attenuated expression in coryneform bacteria. The vector pXK99Emob is an E. coli expression vector and can be employed in E. coli for the enhanced expression of a gene.

Since the vector cannot replicate independently in coryneform bacteria, it is preserved in the cell only when it integrates into the chromosome. The peculiarity of this vector in this connection is the use for the regulated expression of a gene after cloning of a gene segment from the anterior region of the corresponding gene into the

vector, containing the start codon and the native ribosome binding site, and after subsequent integration of the vector in coryneform bacteria, in particular C. glutamicum. By addition of metered amounts of IPTG to the nutrient medium the gene expression is regulated. In this connection, quantities from 0.5 µM up to 10 µM IPTG bring about a very weak expression of the corresponding gene, and quantities from 10 µM up to 100 µM bring about a slightly attenuated to normal expression of the corresponding gene.

The constructed E. coli expression vector pXK99Emob is transferred by means of electroporation (Tauch et al. 1994, FEMS Microbiol Letters, 123: 343-347) into E. coli DH5αmcr (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649). Selection of the transformants is undertaken on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) that has been supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant in accordance with the customary methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 - 927), is cut with the restriction endonuclease NcoI, and the plasmid is examined by subsequent agarose-gel electrophoresis.

The plasmid construct that is obtained in this way is designated as pXK99Emob (Figure 1). The strain that is obtained by electroporation of the plasmid pXK99Emob into the E. coli strain DH5αmcr is called E. coli DH5αmcr/pXK99Emob.

3.3 Cloning of the pfkB fragment into the E. coli expression vector pXK99Emob

By way of vector, use is made of the E. coli expression vector pXK99Emob described in Example 3.2. DNA of this plasmid is cleaved completely with the restriction enzymes

XbaI and HindIII and is subsequently dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, Product No. 1758250).

The approximately 580-bp pfkB fragment described in Example 3.1, which is obtained by means of PCR and cleaved with the restriction endonucleases XbaI and HindIII, is mixed with the prepared vector pXK99Emob, and the charge is treated with T4-DNA-Ligase (Amersham Pharmacia, Freiburg, Germany, product description T4-DNA-Ligase, Code No. 27-0870-04). The ligation charge is transformed into the E. coli strain DH5αmcr (Hanahan, In: DNA Cloning. A Practical Approach, Vol. I, IRL Press, Oxford, Washington DC, USA). Selection of plasmid-bearing cells is undertaken by plating the transformation charge onto LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones are selected. Plasmid DNA is isolated from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and is cleaved with the restriction enzymes XbaI and HindIII, in order to examine the plasmid by subsequent agarose-gel electrophoresis. The plasmid that is obtained is called pXK99EmobpfkB. It is represented in Figure 2.

Example 4

Integration of the vector pXK99EmobpfkB into the genome of the C. glutamicum strain DSM5715

The vector pXK99EmobpfkB named in Example 3 is electroporated into the strain C. glutamicum DSM5715 in accordance with the electroporation method of Tauch et al. (1989 FEMS Microbiology Letters 123: 343-347). The vector cannot replicate independently in DSM5715 and is preserved in the cell only when it has integrated into the

chromosome. Selection of clones with integrated pXK99EmobpfkB is undertaken by plating the electroporation charge onto LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, New York, 1989) that has been supplemented with 15 mg/l kanamycin and IPTG (1 mM).

A selected kanamycin-resistant clone that has inserted the plasmid pXK99EmobpfkB named in Example 3 within the chromosomal pfkB gene of DSM5715 is designated as DSM5715::pXK99EmobpfkB.

Example 5

Preparation of lysine

The C. glutamicum strain DSM5715::pCXK99EmobpfkB obtained in Example 4 is cultured in a nutrient medium that is suitable for the production of lysine, and the lysine content in the supernatant of the culture is determined. Addition of IPTG results in an attenuated expression of the pfkB gene, regulated by the trc promoter.

To this end, the strain is firstly incubated for 24 hours at 33°C on agar plate with the appropriate antibiotic (brain/heart agar with kanamycin (25 mg/l) and IPTG (10 µM)). Starting from this agar-plate culture, a preculture is inoculated (10 ml medium in a 100-ml Erlenmeyer flask). The complete medium Cg III is used as medium for the preculture.

Medium Cg III

| | |
|---|---|
| NaCl | 2.5 g/l |
| bacto-peptone | 10 g/l |
| bacto-yeast extract | 10 g/l |
| glucose (autoclaved separately) | 2% (w/v) |

The pH value was adjusted to pH 7.4

To this medium there are added kanamycin (25 mg/l) and IPTG (10 µM). The preculture is incubated on the shaker for 16 hours at 33°C at 240 rpm. A main culture is inoculated from this preculture, so that the initial OD (660 nm) of the main culture amounts to 0.1. The medium MM is used for the main culture.

To this medium there were added kanamycin (25 mg/l) and IPTG (10 µM). The preculture was incubated on the shaker for 16 hours at 33°C at 240 rpm. The OD (660) of the preculture amounted to 14.7. 68 µl from this preculture were inoculated into a main culture, so that the initial OD (660 nm) of the main culture amounted to 0.1. By virtue of the transfer of IPTG-containing medium from the preculture, the IPTG concentration in the main culture amounted to about 0.07 µM/l. The medium MM was used for the main culture.

Medium MM

| | |
|---|---|
| CSL (corn steep liquor) | 5 g/l |
| MOPS (morpholinopropanesulfonic acid) | 20 g/l |
| glucose (autoclaved separately) | 50 g/l |
| salts: | |
| $(NH_4)_2SO_4$ | 25 g/l |
| $KH_2PO_4$ | 0.1 g/l |
| $MgSO_4 * 7 H_2O$ | 1.0 g/l |
| $CaCl_2 * 2 H_2O$ | 10 mg/l |
| $FeSO_4 * 7 H_2O$ | 10 mg/l |
| $MnSO_4 * H_2O$ | 5.0 mg/l |
| biotin (sterilized by filtration) | 0.3 mg/l |
| thiamin * HCl (sterilized by filtration) | 0.2 mg/l |
| leucine (sterilized by filtration) | 0.1 g/l |
| $CaCO_3$ | 25 g/l |

CSL, MOPS and the salt solution are adjusted to pH 7 with ammoniacal liquor and are autoclaved. Subsequently the sterile substrate and vitamin solutions are added, as well as the dry-autoclaved $CaCO_3$.

Culturing is effected in 10 ml volumes in a 100-ml Erlenmeyer flask with baffles. Kanamycin (25 mg/l) is added. Culturing is effected at 33°C and at 80% atmospheric moisture.

After 48 hours the OD at a measuring wavelength of 660 nm is ascertained with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The quantity of lysine that is formed is determined with an amino-acid analyzer manufactured by Eppendorf-BioTronik (Hamburg, Germany) by ion-exchange chromatography and post-column derivation with detection of ninhydrin.

The result of the experiment is represented in Table 1.

Table 1

| Strain | OD (660 nm) | Lysine HCl g/l |
|---|---|---|
| DSM5715 | 12.2 | 15.31 |
| DSM5715::pXK99EmobpfkB | 7.8 | 16.89 |

Brief description of the Figures:

Figure 1: map of the plasmid pXK99Emob,

Figure 2: map of the plasmid pXK99EmobpfkB.

The abbreviations and designations that are used have the following significance.

| | |
|---|---|
| Kan: | kanamycin-resistance gene aph(3')-IIa from Escherichia coli |
| HindIII | cleavage site of the restriction enzyme HindIII |
| NcoI | cleavage site of the restriction enzyme NcoI |
| XbaI | cleavage site of the restriction enzyme XbaI |
| RP4-mob | RP4 mobilization site |
| Ptrc | trc promoter |
| T1 | termination region T1 |
| T2 | termination region T2 |
| lacIq | lacIq repressor of the lac operon of Escherichia coli |
| oriV | replication origin ColE1 from E. coli |
| pfkB | cloned region of the pfkB gene |

**What is claimed is:**

1. A polynucleotide isolated from coryneform bacteria, containing elongated polynucleotide sequences coding for 1-phosphofructokinase and/or 6-phosphofructokinase, wherein said sequences are each elongated in front of the start codon and behind the stop codon of the gene, in each instance by up to about 700 base-pairs.

2. Isolated polynucleotide according to Claim 1, containing (a) polynucleotide sequence(s) coding for 1-phosphofructokinase and/or 6-phosphofructokinase, wherein said sequence(s) are elongated in front of the start codon and behind the stop codon of the gene, in each instance by up to about 700 base-pairs, the elongated amino-acid sequences being represented in SEQ ID No. 3 for the 1-phosphofructokinase gene and in SEQ ID No. 1 for the 6-phosphofructokinase gene and the elongations in comparison with the sequences known from the state of the art consisting in SEQ ID No. 3 of base-pairs 1 to 508 and 1684 to 2234 and in SEQ ID No. 1 of base-pairs 1 to 531 and 1621 to 2160.

3. A process for the fermentative preparation of L-amino acids, in particular L-lysine, wherein the following steps are implemented:

   a) fermentation of the coryneform bacteria producing the desired L-amino acid, in which at least the gene coding for 6-phosphofructokinase and/or the gene coding for 1-phosphofructokinase are/is attenuated,

   b) enrichment of the desired product in the medium or in the cells of the bacteria, and

   c) isolation of the desired L-amino acid, whereby constituents of the fermentation broth and/or of

the biomass optionally remain in the end product in proportions or in their total quantities.

4. Process according to Claim 3, wherein coryneform bacteria are employed in which the attenuation is achieved by using the polynucleotide sequences that are elongated in front of the start codon and behind the stop codon of the respective gene by, in each instance, 300 to 800 base-pairs.

5. Process according to Claim 4, wherein coryneform bacteria are employed in which the attenuation is achieved by using the polynucleotide sequences that are elongated in front of the start codon and behind the stop codon of the gene, in each instance by about 700 base-pairs, the elongated nucleotide sequences being represented in SEQ ID No. 3 for the 1-phosphofructokinase gene and in SEQ ID No. 1 for the 6-phosphofructokinase gene and the elongations in SEQ ID No. 3 in comparison with the sequence known from the state of the art consisting of base-pairs 1 to 508 and 1684 to 2234 and in SEQ ID No. 1 in comparison with the sequence known from the state of the art consisting of base-pairs 1 to 531 and 1621 to 2160.

6. Process according to Claim 3, wherein bacteria are employed in which, in addition, further genes of the biosynthetic pathway of the desired L-amino acid are enhanced.

7. Process according to Claim 3, wherein bacteria are employed in which the metabolic pathways that diminish the formation of the desired L-amino acid are at least partially switched off.

8. Process according to Claim 3, wherein the expression of the polynucleotide(s) that codes/code for 6-

phosphofructokinase and/or for 1-phosphofructokinase is diminished.

9. Process according to Claim 3, wherein the catalytic properties of the polypeptide(s) (enzyme protein(s)) for which the polynucleotide(s) from SEQ ID NO. 1 and SEQ ID No. 3 codes/code are reduced.

10. Process according to Claim 3, wherein for the preparation of L-lysine coryneform micro-organisms are fermented in which simultaneously one or more of the genes selected from the group comprising

10.1 the gene lysC coding for a feedback-resistant aspartate kinase,

10.2 the gene dapA coding for dihydrodipicolinate synthase,

10.3 the gene gap coding for glyceraldehyde-3-phosphate dehydrogenase,

10.4 the gene pyc coding for pyruvate carboxylase,

10.5 the gene mqo coding for malate:quinone oxidoreductase,

10.6 the gene zwf coding for glucose-6-phosphate dehydrogenase,

10.7 simultaneously the gene lysE coding for lysine export,

10.8 the gene zwa1 coding for the zwa1 protein,

10.9 the gene tpi coding for triosephosphate isomerase, and

10.10 the gene pgk coding for 3-phosphoglycerate kinase

is/are enhanced, in particular overexpressed.

11. Process according to Claim 3, wherein with a view to preparing L-amino acids coryneform micro-organisms are fermented in which simultaneously one or more of the genes selected from the group comprising

11.1 the pck gene coding for phosphoenolpyruvate carboxykinase,

11.2 the pgi gene coding for glucose-6-phosphate isomerase,

11.3 the gene poxB coding for pyruvate oxidase,

11.4 the gene fda coding for fructose bisphosphate aldolase, and

11.5 the gene zwa2 coding for the zwa2 protein

is/are attenuated.

12. Process according to one or more of Claims 3 to 11, wherein micro-organisms of the species Corynebacterium glutamicum are employed.

13. Coryneform bacteria in which at least the gene coding for 6-phosphofructokinase and/or the gene coding for 1-phosphofructokinase are/is present in attenuated form.

14. Escherichia coli strain DH5alphamcr/pXK99EmobpfkB (= DH50mcr/ pXK99EmobpfkB), deposited as DSM 14741 in the Deutschen Sammlung für Mikroorganismen und Zellkulturen (German Collection of Micro-Organisms and Cell Cultures), Braunschweig, Germany.

Figure 1: Plasmid pXK99Emob

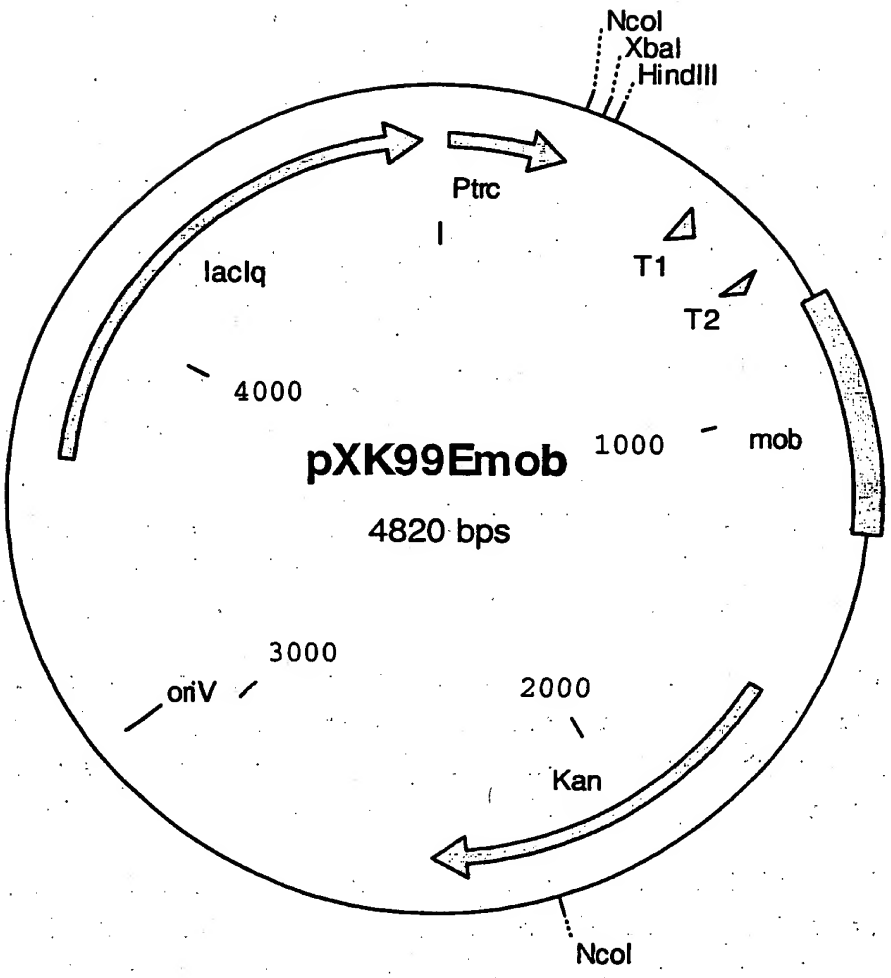
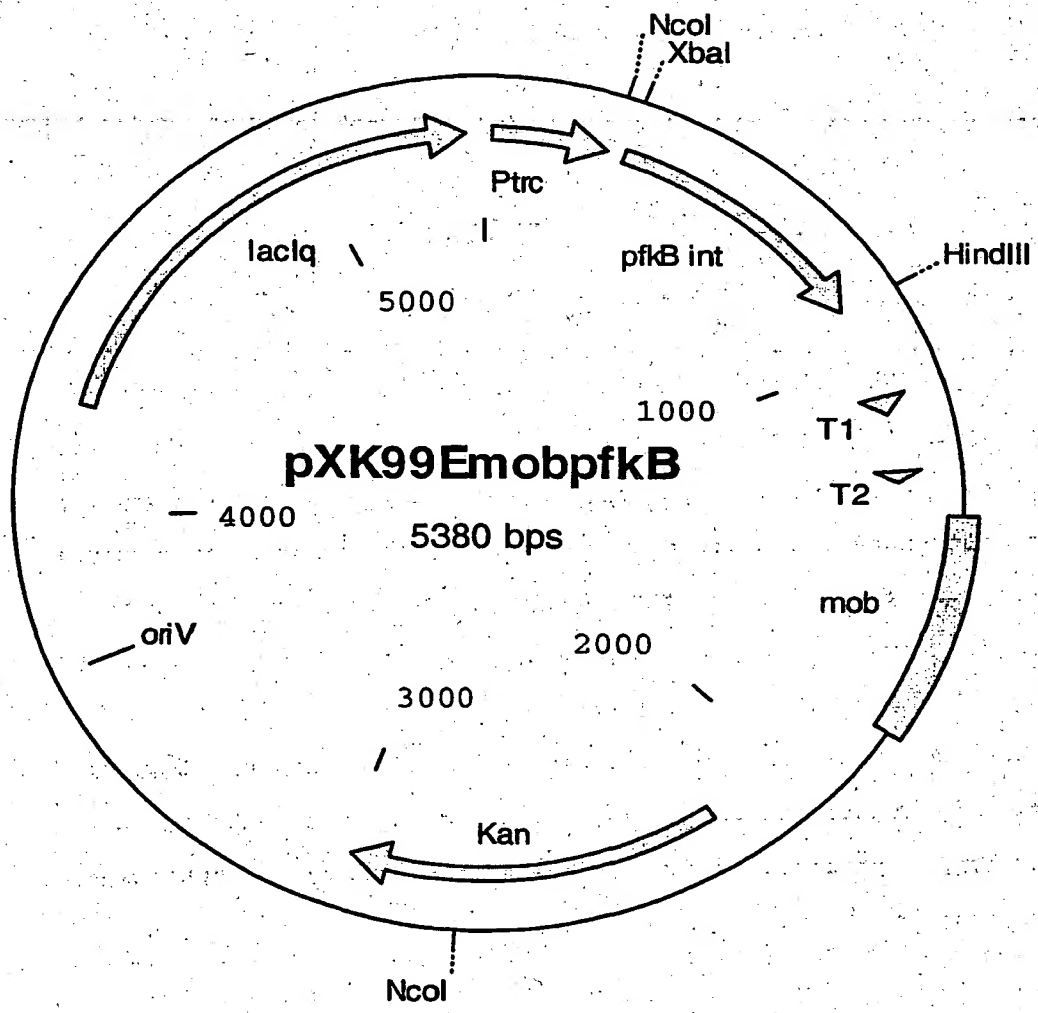


Figure 2: pXK99EmobpfkB



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Gly Trp Glu Gly Leu Leu Gly Asp Arg Arg Val Gln Leu Tyr Asp Asp
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Gly Arg Leu His Pro Asp Lys Phe Lys Ala Gly Ile Asp Gln Ile Lys
                75                  80                  85

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Ala Asn Leu Glu Asp Ala Gly Ile Asp Ala Leu Ile Pro Ile Gly Gly
            90                  95                  100

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Gln Phe Gly His Lys Thr Phe Thr Gly Ile Gly Gln Gln Ile Ala Asp
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gtaacccc atg atc atc aca ttc acc cca aac ccg agt att gat tcc acg      650
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ctg tcg ctc ggc gaa gag ctc tcc cgt gga tcc gtc caa cga ctt gat      698
Leu Ser Leu Gly Glu Glu Leu Ser Arg Gly Ser Val Gln Arg Leu Asp
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tcc gtc acc gct gtc gca ggt ggt aaa ggc atc aat gtc gcc cac gct      746
Ser Val Thr Ala Val Ala Gly Gly Lys Gly Ile Asn Val Ala His Ala
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gtc ttg ctt gcg ggc ttt gaa acc ttg gct gtg ttc cca gcc ggc aag      794
Val Leu Leu Ala Gly Phe Glu Thr Leu Ala Val Phe Pro Ala Gly Lys
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ctc gac ccc ttc gtc cca ctg gtc cgc gac atc ggc ttg ccc gtg gaa      842
Leu Asp Pro Phe Val Pro Leu Val Arg Asp Ile Gly Leu Pro Val Glu
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act gtt gtg atc aac aag aac gtc cgc acc aac acc aca gtc acc gaa      890
Thr Val Val Ile Asn Lys Asn Val Arg Thr Asn Thr Thr Val Thr Glu
    80                  85                  90

ccg gac ggc acc acc acc aag ctc aac ggc ccc ggc gcg ccg ctc agc      938
Pro Asp Gly Thr Thr Thr Lys Leu Asn Gly Pro Gly Ala Pro Leu Ser
95                  100                 105                 110

gag cag aag ctc cgt agc ttg gaa aag gtg ctt atc gac gcg ctc cgc      986
Glu Gln Lys Leu Arg Ser Leu Glu Lys Val Leu Ile Asp Ala Leu Arg
                115                 120                 125

ccc gaa gtc acc tgg gtt gtc ctg gcg ggc tcg ctg cca cca ggg gca     1034
Pro Glu Val Thr Trp Val Val Leu Ala Gly Ser Leu Pro Pro Gly Ala
            130                 135                 140

cca gtt gac tgg tac gcg cgt ctc acc gcg ttg atc cat tca gca cgc     1082
Pro Val Asp Trp Tyr Ala Arg Leu Thr Ala Leu Ile His Ser Ala Arg
        145                 150                 155

cct gac gtt cgc gtg gct gtc gat acc tca gac aag cca ctg atg gcg     1130
Pro Asp Val Arg Val Ala Val Asp Thr Ser Asp Lys Pro Leu Met Ala
    160                 165                 170

ttg ggc gag agc ttg gat aca cct ggc gct gct ccg aac ctg att aag     1178
Leu Gly Glu Ser Leu Asp Thr Pro Gly Ala Ala Pro Asn Leu Ile Lys
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cca aat ggt ctg gaa ctg ggc cag ctg gct aac act gat ggt gaa gag      1226
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ctg gag gcg cgt gct gcg caa ggc gat tac gac gcc atc atc gca gct      1274
Leu Glu Ala Arg Ala Ala Gln Gly Asp Tyr Asp Ala Ile Ile Ala Ala
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gcg gac gta ctg gtt aac cgt ggc atc gaa cag gtg ctt gtc acc ttg      1322
Ala Asp Val Leu Val Asn Arg Gly Ile Glu Gln Val Leu Val Thr Leu
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ggt gcc gca gga gcg gtg ttg gtc aac gca gaa ggt gcg tgg act gct      1370
Gly Ala Ala Gly Ala Val Leu Val Asn Ala Glu Gly Ala Trp Thr Ala
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Thr Ser Pro Lys Ile Asp Val Val Ser Thr Val Gly Ala Gly Asp Cys
255                 260                 265                 270

gct ctt gca ggt ttt gtt atg gca cgt tcc cag aag aaa aca ctg gag      1466
Ala Leu Ala Gly Phe Val Met Ala Arg Ser Gln Lys Lys Thr Leu Glu
                275                 280                 285

gaa tct ctg ctg aat gcc gtg tct tac ggc tcg act gcg gcg tct ctt      1514
Glu Ser Leu Leu Asn Ala Val Ser Tyr Gly Ser Thr Ala Ala Ser Leu
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Pro Gly Thr Thr Ile Pro Arg Pro Asp Gln Leu Ala Thr Ala Gly Ala
        305                 310                 315

acg gtc acc caa gtc aaa gga ttg aaa gaa tca gca tgaatagcgt           1608
Thr Val Thr Gln Val Lys Gly Leu Lys Glu Ser Ala
    320                 325                 330

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Thr Ala Val Ala Gly Gly Lys Gly Ile Asn Val Ala His Ala Val Leu
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Leu Ala Gly Phe Glu Thr Leu Ala Val Phe Pro Ala Gly Lys Leu Asp
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Pro Phe Val Pro Leu Val Arg Asp Ile Gly Leu Pro Val Glu Thr Val
65                  70                  75                  80

Val Ile Asn Lys Asn Val Arg Thr Asn Thr Thr Val Thr Glu Pro Asp
                85                  90                  95

Gly Thr Thr Thr Lys Leu Asn Gly Pro Gly Ala Pro Leu Ser Glu Gln
            100                 105                 110

Lys Leu Arg Ser Leu Glu Lys Val Leu Ile Asp Ala Leu Arg Pro Glu
        115                 120                 125

Val Thr Trp Val Val Leu Ala Gly Ser Leu Pro Pro Gly Ala Pro Val
    130                 135                 140

Asp Trp Tyr Ala Arg Leu Thr Ala Leu Ile His Ser Ala Arg Pro Asp
145                 150                 155                 160

Val Arg Val Ala Val Asp Thr Ser Asp Lys Pro Leu Met Ala Leu Gly
                165                 170                 175

Glu Ser Leu Asp Thr Pro Gly Ala Ala Pro Asn Leu Ile Lys Pro Asn
            180                 185                 190

Gly Leu Glu Leu Gly Gln Leu Ala Asn Thr Asp Gly Glu Glu Leu Glu
        195                 200                 205

Ala Arg Ala Ala Gln Gly Asp Tyr Asp Ala Ile Ile Ala Ala Ala Asp
    210                 215                 220

Val Leu Val Asn Arg Gly Ile Glu Gln Val Leu Val Thr Leu Gly Ala
225                 230                 235                 240

Ala Gly Ala Val Leu Val Asn Ala Glu Gly Ala Trp Thr Ala Thr Ser
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Pro Lys Ile Asp Val Val Ser Thr Val Gly Ala Gly Asp Cys Ala Leu
            260                 265                 270

Ala Gly Phe Val Met Ala Arg Ser Gln Lys Lys Thr Leu Glu Glu Ser
        275                 280                 285

Leu Leu Asn Ala Val Ser Tyr Gly Ser Thr Ala Ala Ser Leu Pro Gly
    290                 295                 300

Thr Thr Ile Pro Arg Pro Asp Gln Leu Ala Thr Ala Gly Ala Thr Val
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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2

33970 Halle/Künsebeck

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

| I. IDENTIFICATION OF THE MICROORGANISM | |
|---|---|
| Identification reference given by the DEPOSITOR: DH5alphamcr/ pXK99EmobpfkB | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 14741 |

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(X) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

The International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-01-11 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

| | |
|---|---|
| Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH<br>Address: Mascheroder Weg 1b, D-38124 Braunschweig | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Wehs<br>Date: 2002-01-14 |

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 0196

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2

33970 Halle/Künsebeck

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

| I. DEPOSITOR | II. IDENTIFICATION OF THE MICROORGANISM |
|---|---|
| Name: Degussa AG<br>Kantstr. 2<br>Address:<br>33970 Halle/Künsebeck | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:<br>DSM 14741<br>Date of the deposit or the transfer[1]:<br>2002-01-11 |

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2002-01-11 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

| | |
|---|---|
| Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH<br>Address: Mascheroder Weg 1b<br>D-38124 Braunschweig | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):<br>U. Weiß<br>Date: 2002-01-14 |

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
3 Mark with a cross the applicable box.
4 Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 0196

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/02830

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/12 C12N15/54 C12P13/04 C12P13/08 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBL, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X<br>P,Y | EP 1 103 613 A (DEGUSSA AG)<br>30 May 2001 (2001-05-30)<br>See SEQ ID No. 1<br>the whole document | 1,2<br>3-14 |
| P,X<br>P,Y | EP 1 106 622 A (DEGUSSA AG)<br>13 June 2001 (2001-06-13)<br>see SEQ ID No. 1<br>the whole document | 1,2<br>3-14 |
| P,X | EP 1 108 790 A (KYOWA HAKKO KOGYO CO., LTD.) 20 June 2001 (2001-06-20)<br>see SEQ ID No. 7063 and SEQ ID 7065<br>the whole document | 1,2 |

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 1 July 2002 | 11/07/2002 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Douschan, K |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/02830

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | PATENT ABSTRACTS OF JAPAN<br>vol. 012, no. 344 (C-528),<br>16 September 1988 (1988-09-16)<br>& JP 63 102692 A (KYOWA HAKKO KOGYO CO LTD), 7 May 1988 (1988-05-07)<br>abstract | 1-14 |
| Y | WO 00 77172 A (AJINOMOTO CO., INC.)<br>21 December 2000 (2000-12-21)<br>see the abstract | 1-14 |

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[illegible]ional Application No
PCT/EP 02/02830

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|---|
| EP 1103613 | A | 30-05-2001 | DE | 19956131 A1 | 31-05-2001 |
| | | | AU | 7166500 A | 24-05-2001 |
| | | | BR | 0005543 A | 07-08-2001 |
| | | | CN | 1297055 A | 30-05-2001 |
| | | | EP | 1103613 A1 | 30-05-2001 |
| | | | JP | 2001186895 A | 10-07-2001 |
| | | | PL | 344076 A1 | 04-06-2001 |
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| EP 1106622 | A | 13-06-2001 | DE | 10011922 A1 | 31-05-2001 |
| | | | AU | 7174500 A | 24-05-2001 |
| | | | BR | 0005531 A | 07-08-2001 |
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| | | | EP | 1106622 A2 | 13-06-2001 |
| | | | JP | 2001186896 A | 10-07-2001 |
| | | | PL | 344073 A1 | 04-06-2001 |
| | | | SK | 17372000 A3 | 03-12-2001 |
| EP 1108790 | A | 20-06-2001 | EP | 1108790 A2 | 20-06-2001 |
| JP 63102692 | A | 07-05-1988 | JP | 2101579 C | 22-10-1996 |
| | | | JP | 7121227 B | 25-12-1995 |
| WO 0077172 | A | 21-12-2000 | AU | 5106800 A | 02-01-2001 |
| | | | BR | 0011672 A | 19-03-2002 |
| | | | EP | 1195431 A1 | 10-04-2002 |
| | | | WO | 0077172 A1 | 21-12-2000 |

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